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## PURIFICATION OF 45KDa ESTRAMUSTINE BINDING PROTEIN (EMBP) AND PREPARATION OF ITS ANTIBODY

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The purification of 45KDa EMBP and the production of monospecific anti-serum is described. 45KDa EMBP was purified by relatively simple methods using ion exchange HPLC (TSK-GEL DEAE-5PW column) and size exclusion HPLC (TSK-GEL G3000SW column). The results clearly demonstrated the speed and simplicity of the method using these columns, compared to previously-described methods for purification of 45KDa EMBP.

**Key words:** 45KDa estramustine binding protein, Rat prostate

### INTRODUCTION

Estramustine binding protein (EMBP) was discovered during investigation of estramustine phosphate (Estracyt, estradiol-3N-bis-(2-chloroethyl) carbamate-17 $\beta$ -phosphate), a drug used for the treatment of prostate cancer<sup>1-3)</sup>. This protein may be identical to a protein studied by several investigators, called  $\alpha$ -protein by Liao et al.<sup>4)</sup>, prostatic binding protein by Heyns et al.<sup>5,6)</sup> and prostatein by Lea et al.<sup>7)</sup>. Although the biological function of this protein is not clear, it can bind non-polar steroids<sup>1,5)</sup>, cholesterol<sup>8)</sup>, proline-rich polypeptides<sup>9)</sup> and chemical carcinogens<sup>10)</sup>. Study of this specific protein in detail requires purification of 45KDa EMBP and production of a monospecific anti-serum.

### MATERIALS AND METHODS

Chemicals and radiochemical compounds: <sup>3</sup>H-estramustine [2, 4, 6, 7-<sup>3</sup>H (N)-estradiol-17 $\beta$ -3N-bis-(2-chloroethyl) carbamate: 105 Ci/mmole] and estramustine were kindly supplied by AB Leo, Helsingborg, Sweden.

Animals: Male Sprague-Dawley strain rats were purchased from Japan Charles River Co. at 9-10 weeks of age.

**Purification of 45KDa EMBP:** The prostates of 9- to 10-week-old rats were removed, immediately frozen in liquid nitrogen and stored at -80°C until use. The frozen prostates were pulverized and homogenized in about 3 volumes of a buffer containing 10 mM Tris-HCl (pH 7.2), 1 mM EDTA, 0.1 mM dithiothreitol and 10% glycerol (TEDG buffer), using a Polytron PT (Kinematica Switzerland). The homogenate was centrifuged for 60 min at 105,000 g in a Hitachi RP 80T rotor and the supernatant (cytosol) was carefully separated. The supernatant was applied to an ion exchange HPLC (TSK-GEL DEAE-5PW column). The elution was performed at 1 ml per minute with a 30-minute linear gradient from 50 mM Tris-HCl (pH 7.2) buffer to 0.4 M sodium chloride-containing 50 mM Tris-HCl (pH 7.2) buffer. The fractions containing a sharp radioactive peak were combined and injected into a size exclusion HPLC (TSK-GEL G3000SW). The elution was performed at 1 ml per minute using 0.075 M phosphate buffer (pH 7.2).

**Immunization:** Two rabbits were immunized with the purified 45KDa EMBP. The protein, 1 mg in 0.5 ml of phosphate buffer solution, was mixed with 0.6 ml of

Freund complete adjuvant and injected intradermally at multiple sites. Booster injections were given twice a month after the first injection. The rabbits were bled from an ear vein 6 weeks after the first injection and bled out 8 weeks later. IgG was purified from rabbit anti-rat 45-KDa EMBP serum using an ion exchange HPLC (TSK-GEL DEAE-5PW column).

**Polyacrylamide gel electrophoresis:** Electrophoresis was performed in 7 % acrylamide gel, without SDS, in system No. 3 of Maurer & Allen<sup>11)</sup> or in 15 % gel containing 0.1% SDS, as described by Laemmli<sup>12)</sup>. Before SDS polyacrylamide gel electrophoresis, the samples were boiled with 2% SDS, with or without  $\beta$ -mercaptoethanol. The gels were stained for protein with Coomassie Brilliant Blue R 250.

**Analysis of amino acid composition:** About 200  $\mu$ g of purified 45KDa EMBP was hydrolysed with 12N HCl and analyzed for amino acid composition at Yanaco Laboratory, Kyoto, Japan.

**Protein and carbohydrate measurement:** Protein was measured using the method of Lowry et al.<sup>13)</sup>, with BSA as a standard. Carbohydrate was measured by the orcinol-sulfuric acid method<sup>14)</sup>.

## RESULTS

**Purification of 45KDa EMBP from the rat ventral prostate:** Fig. 1-(1) shows the elution profile of rat ventral prostate cytosol incubated with  $^3$ H-estramustine on an ion exchange HPLC, which is the first step of 45KDa EMBP purification. Two radioactive peaks were seen (peak A and peak B). Peak B corresponded to the sharp protein peak at 280 nm. The fractions corresponding to peak B were combined, and were applied to a TSK-GEL G3000SW column (the second step of purification). In the eluate of the size exclusion HPLC there was a striking correlation between the binding activity and the protein peak (Fig. 1-(2)). This main radioactive peak corresponded to the protein peak with a molecular weight of 45,000. The top fractions of this peak were combined and used for immunization.

**Polyacrylamide gel electrophoresis and amino acid composition of the purified 45KDa EMBP:** Polyacrylamide gel electrophoresis of the purified 45KDa EMBP, in the absence of sodium dodecylsulphate and disulfide bridge reducing agent in system No. 3 of Maurer & Allen, showed a single protein band, indicating a homogeneous preparation of the protein

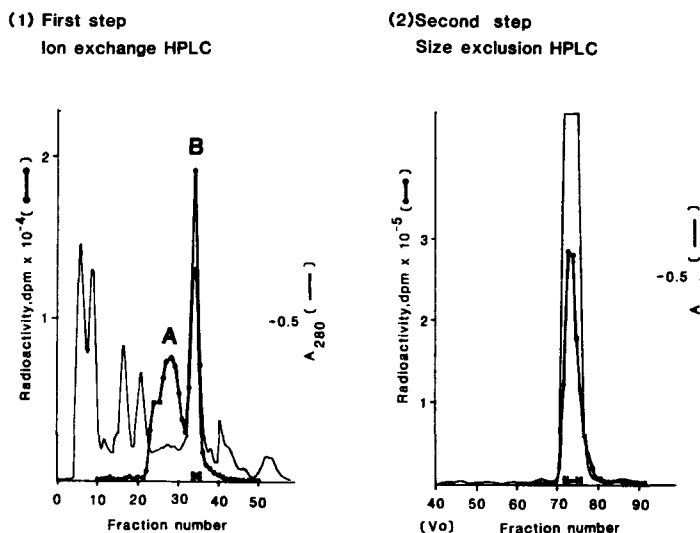


Fig. 1. Purification of 45KDa EMBP from the rat ventral prostate

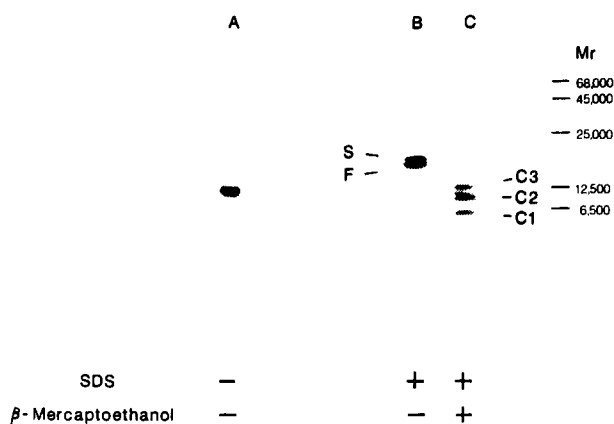


Fig. 2. Polyacrylamide gel electrophoresis of the purified 45KDa EMBP. Gels A and B were composed of purified 45KDa EMBP analyzed in the absence and presence, respectively, of SDS (samples were not treated with  $\beta$ -mercaptoethanol). Gel C contained SDS and the sample was treated with SDS and  $\beta$ -mercaptoethanol. The indicated molecular weights corresponded to the following marker proteins: bovine serum albumin (68,000), egg albumin (45,000), chymotrypsinogen A (25,000), cytochrome C (12,500) and aprotinin (6,500). Proteins migrated from the top of the gels toward the anode at the bottom of the gels.

(Fig. 2-A). Analysis by SDS-polyacrylamide gel electrophoresis showed that 45KDa EMBP dissociated into two well-separated bands, corresponding to the S (slow) and F (fast) subunits (Fig. 2-B). The molecular weights were about 20,000 for subunit S and 18,000 for subunit F. After reduction, these bands disappeared and were replaced by three bands (components 1, 2 and 3) (Fig. 2-C). The molecular weights of components 1, 2 and 3 were about 6,000, 10,000 and 13,000, respectively. Table 1 shows the amino acid composition of the purified 45KDa EMBP. Sulfur-containing amino acids accounted for about 3% (molar basis) and about 26% of the acidic amino acids. The presence of about 5% carbohydrate indicated that 45KDa EMBP was a glycoprotein.

The reaction between the purified 45KDa EMBP and the IgG fraction of rabbit anti-45KDa EMBP serum: Fig. 3 shows the elution profile of purified 45KDa EMBP labelled with  $^3\text{H}$ -estramustine on an ion exchange HPLC after incubation with normal rabbit serum (control) or an IgG fraction of rabbit anti-45KDa EMBP serum. Incubation with normal rabbit

serum did not change the radioactive peak. After incubation with the IgG fraction of rabbit anti-45KDa EMBP serum, the radioactive peak at 45KDa EMBP disappeared, indicating an immunoreaction between the purified 45KDa EMBP and the rabbit anti-45KDa EMBP serum.

## DISCUSSION

The present investigation demonstrated that 45KDa EMBP from the rat ventral prostate was purified using relatively simple procedures. The present results of polyacrylamide gel electrophoresis coincided with the results of Heyns et al.<sup>6)</sup>. They reported that this protein was a heterodimer consisting of S and F subunits, each subunit contained two disulfide linked polypeptides C3-C2 and C3-C1, respectively. The amino acid composition of the 45KDa EMBP (Table 1) coincided with that described by Gustafsson et al.<sup>15)</sup>. Fig. 3 also indicates that the purified 45KDa EMBP has a reliable binding ability for estramustine. These results clearly demonstrate that the usage of the TSK-GEL DEAE-5PW and TSK-GEL G3000SW columns offered a faster and

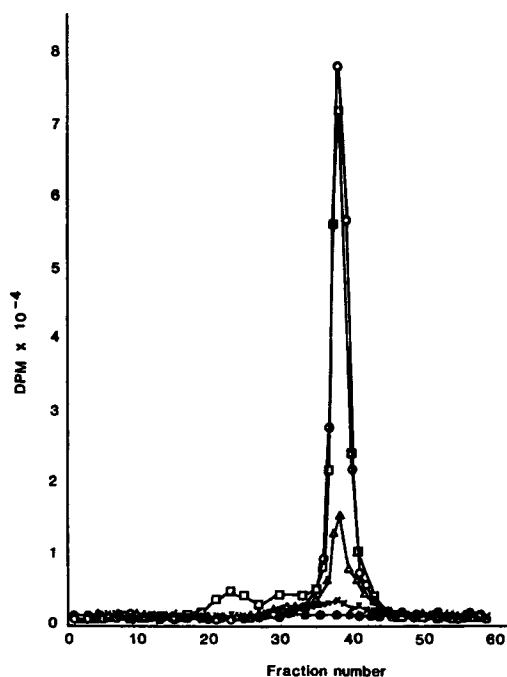


Fig. 3. The reaction between the purified 45 KDa EMBP with IgG fraction of rabbit anti-45KDa EMBP serum. 10 µg of purified 45KDa EMBP dissolved in TEDG buffer was incubated for 3 hours at 0°C with  $1 \times 10^{-8}$  M  $^3$ H-estramustine. Various amounts of IgG fraction of rabbit anti-45KDa EMBP serum were then added to the incubated solution containing 3 µg of the  $^3$ H-estramustine labelled 45KDa EMBP. The fixed amounts of the incubated solution were injected into the ion exchange HPLC on the TSK-GEL DEAE-5PW column. The elution was performed at 1 ml per minute with a 30 minute linear elution gradient from 50 mM Tris-HCl (pH 7.2) buffer to 0.4M sodium chloride-containing 50 mM Tris-HCl (pH 7.2) buffer.

○—○: 3 µg of purified 45 KDa EMBP labelled with  $^3$ H-estramustine

□—□: 3 µg of purified 45KDa EMBP labelled with  $^3$ H-estramustine + rabbit serum

●—●: 3 µg of purified 45 KDa EMBP labelled with  $^3$ H-estramustine + 337.5 µg of IgG fraction of rabbit anti-45KDa EMBP serum

×—×: 3 µg of purified 45 KDa EMBP labelled with  $^3$ H-estramustine + 33.8 µg of IgG fraction of rabbit anti-45KDa EMBP serum

△—△: 3 µg of purified 45 KDa EMBP labelled with  $^3$ H-estramustine + 6.75 µg of IgG fraction of rabbit anti-45KDa EMBP serum

Table 1. The amino acid composition of the purified 45KDa EMBP. About 200 µg of purified 45KDa EMBP was hydrolyzed with 12N HCl and analyzed for amino acid composition at Yanaco Laboratory, Kyoto.

Amino acid	mole %	
Aspartic acid	10.17	( 2 )
Threonine	6.17	
Serine	6.69	
Glutamic acid	15.57	( 1 )
Proline	3.66	
Glycine	4.45	
Alanine	5.93	
Cystine	0.27	
Valine	8.76	( 4 )
Methionine	2.48	
Isoleucine	5.52	
Leucine	9.44	( 3 )
Tyrosine	3.47	
Phenylalanine	5.04	
Lysine	7.77	
Histidine	1.80	
Arginine	3.01	
100.00		

simpler method for purification of 45KDa EMBP than the method previously described by other investigators<sup>1,5,7).</sup>

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## 和文抄録

### 45KDa Estramustine Binding Protein (EMBP) の精製と抗体作成

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われわれは、ラット前立腺腫瘍より 45KDa EMBP を部分精製し、その抗体を作成することをこころみた。精製には、陰イオン交換 HPLC (TSK-GEL DEAE-5PW) およびゲル濾過 HPLC (TSK-GEL G3000SW) をもちい、電気泳動的に単一で、しかも生物活性の高い標品を得ることができた。この

HPLC を用いての 45KDa EMBP 精製方法は従来の方法と比較して、より迅速、より簡便な方法であり、有用性は高いと思われた。また、この抗原を家兎に免疫し、特異性の高い抗体が得られた。

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